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TACI-Ig Neutralizes Molecules Critical for B Cell Development and Autoimmune Disease: Impaired B Cell Maturation in Mice Lacking BLyS

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Summary

BLyS and APRIL have similar but distinct biological roles, mediated through two known TNF receptor family members, TACI and BCMA. We show that mice treated with TACI-Ig and TACI-Ig transgenic mice have fewer transitional T2 and mature B cells and reduced levels of circulating immunoglobulin. TACI-Ig treatment inhibits both the production of collagen-specific Abs and the progression of disease in a mouse model of rheumatoid arthritis. In *BLyS*-deficient mice, B cell development is blocked at the transitional T1 stage such that virtually no mature B cells are present, while B-1 cell numbers are relatively normal. These findings further elucidate the roles of BLyS and APRIL in modulating B cell development and suggest that BLyS is required for the development of most but not all mature B cell populations found in the periphery.

Introduction

Development of the mature B cell repertoire from bone marrow (BM) precursors is a complex process governed by a variety of molecular and cellular events (Hayakawa et al., 1997; Yankee and Clark, 2000). After successful expression of surface immunoglobulin (Ig), immature B cells are subjected to a series of signaling events through the BCR (DeFranco, 1997), local receptors, and factors from the microenvironment, that ultimately determine their developmental fate (Loder et al., 1999). These signals serve as checkpoints to ensure that a fully competent B cell repertoire, capable of reacting to a diverse set of antigens, is achieved while maintaining tolerance to self-antigens (Melchers et al., 1995).

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The mature B cell pool consists predominantly of two functionally and phenotypically distinct populations: conventional B-2 B cells located in the follicles and marginal zones (MZ) (Oliver et al., 1997) as well as B-1 B cells located in peritoneal cavities (Hayakawa and Hardy, 2000). Immature B cells exit the BM as transitional T1 B cells and migrate to the periarterial lymphatic sheath (PALS). These cells are thought to undergo selection processes mediated by signals through the BCR and from the microenvironment (Loder et al., 1999). T1 cells are thought to differentiate into transitional T2 B cells, acquiring expression of CD23 and IgD (Loder et al., 1999). MZ B cells, located in the space surrounding the follicle, represent a type of memory B cell capable of responding rapidly to antigenic challenge involving local metallophilic macrophages and dendritic cells (Martin and Kearney, 2000b). The mature B cell repertoire also includes B-1 B cells that produce the majority of Abs to self-antigens (Arnold et al., 1994; Hardy and Hayakawa, 2001). The development of B-1 and B-2 cells involves both positive and negative selection events, modulated in part by the strength of BCR-mediated signals. Additional environmental signals and migration events also contribute to the developmental regulation of both B cell lineages (Hardy et al., 1994; Melchers et al., 1995).

BLyS (BAFF, TALL-1, THANK, zTNF4) and APRIL are molecules that modulate B cell activation (reviewed in Laabi and Strasser, 2000), survival (Batten et al., 2000; Do et al., 2000; Thompson et al., 2000), and development (Gross et al., 2000; Mackay et al., 1999). A unique property of APRIL is its ability to costimulate T cells in vitro and to promote tumor survival (Hahne et al., 1998; Rennert et al., 2000; Yu et al., 2000). In vivo, BLyS overexpression in transgenic (TG) mice results in the expansion of mature B-2 and B-1 B cells in the spleen and symptoms characteristic of autoimmune disease (Gross et al., 2000, Mackay et al., 1999). Both BLyS and APRIL bind TACI and BCMA and are thought to function through these receptors (Gross et al., 2000; Marsters et al., 2000; Wu et al., 2000). TACI is expressed on mature B cells and on activated T cells (von Bulow and Bram, 1997), whereas BCMA expression is restricted to B cell populations (Laabi and Strasser, 2000; Madry et al., 1998). However, it is not yet clear precisely where in B cell development these receptors function. Soluble receptor forms of TACI and BCMA can inhibit BLyS and APRIL activity in vitro (Gross et al., 2000; Marsters et al., 2000; Yu et al., 2000). In vivo, TACI-Ig can inhibit the levels of circulating $B220^+$ B cells (Gross et al., 2000), antibody (Ab) production to a T-dependent antigen (Yan et al., 2000; Yu et al., 2000), and the development of disease in mice that spontaneously develop symptoms characteristic of systemic lupus erythematosus (SLE) (Gross et al., 2000).

Here, we demonstrate that the TACI-Ig soluble receptor can block the development of specific B cell populations in the periphery, suggesting a role for the factors neutralized by TACI-Ig (including BLyS and APRIL) in supporting B cell homeostasis. We also investigate the

ability of TACI-Ig to inhibit the development of collagen-specific Abs and the incidence of disease in a mouse model of collagen-induced arthritis (CIA). Last, we produced *BLyS* $^{-/-}$ mice to dissect the role of this ligand in the development of B cells in the preimmune mouse. These results provide compelling data indicating the importance of factors delivered by specific microenvironments in the development of normal and autoreactive B cells.

Results

TACI-Ig Treatment of Naive Mice Results in a Specific Loss of Mature B Cell Populations but Has Little Effect on Other Cell Lineages

We treated naive mice with 100 μg of human TACI-Ig, human IgG (Hu-Ig), or PBS three times a week for a total of 2 weeks to determine its effect on B cells and on other cells of the immune system. There was no significant difference in the total cell numbers found in spleen, thymus, peritoneal exudates (PEC), mesenteric lymph node (mLN), or BM of mice treated with TACI-Ig (data not shown). Cells from each tissue were stained with fluorescently labeled mAbs and analyzed by immunofluorescence flow cytometry (FACS) in order to identify specific cell lineages in each of these organs (Figure 1A). TACI-Ig treatment had no effect on the percentages or total numbers of immature and mature thymocytes (data not shown), of mature T cells defined by CD4/CD8 surface expression in the thymus and spleen (data not shown), or of $CD5^+B220^-$ T cells in mLN, spleen, or PEC (Figure 1A). Spleen monocyte populations identified either as $CD5^-B220^-$ (Figure 1A) or as $CD14^+$ cells (data not shown) were not affected significantly by TACI-Ig treatment.

However, TACI-Ig had a profound effect on the representation of B cells in peripheral lymphoid organs after treatment with TACI-Ig for 14 days. The average percentage of B cells defined as $CD5^-B220^+$ in the mLN of untreated mice was 27.9 ± 1.6% versus 13.4 ± 1.8% in treated mice and dropped from 52.5 ± 3.7% to 36.5 ± 2.0%, respectively, in the spleen (Figure 1A). There also was a moderate decrease in the percentage of B-1 ($B220^+CD5^+$) B cells in the PEC of TACI-Ig treated mice, dropping from 38.3 ± 2.1% in the control group to 29.5 ± 1.4% in the TACI-Ig treated mice (Figure 1A). The BM was analyzed to determine the effect of TACI-Ig treatment on stem cell populations and on B cell precursors. There was no apparent change in the fraction of BM-derived stem cells in the $B220^-IgM^-IgD^-$ population (Figure 1A). In addition, $B220^+$ pre- and pro-B cells ($B220^+IgM^-IgD^-$) and immature B cells ($B220^-IgM^+IgD^-$) were not altered by TACI-Ig treatment. However, there was a clear decrease in the mature recirculating B cell population ($B220^+IgM^+IgD^+$) in the BM. The average percentage of these cells dropped from 10.5 ± 3.2% in the group treated with Hu-Ig to 5.6 ± 1.3% in the TACI-Ig treatment group (Figure 1A).

TACI-Ig Specifically Blocks the Development of B Cells at the Transition from T1 to T2 and the Development of MZ B Cells in the Spleen

We examined the numbers of splenic B cell populations (defined as $B220^+$) to determine which populations were affected by treatment with TACI-Ig versus Hu-Ig or PBS (Figure 1B). We used Abs to CD21, CD23, IgD, and IgM to distinguish transitional T1 (IgM^+CD21^-) from T2 cells and MZ (IgM^+CD21^+) B cells (Figure 1). There was a clear block in development of both T2 and MZ B cells after exposure to TACI-Ig. There was a slight increase in newly formed (NF) B cells, defined as $B220^+CD21^-CD23^-$ (Figure 1B). However, there was no incorporation of 5-bromo-2′-deoxyuridine (BrdU) within the NF B cell population in the TACI-Ig treated mice (data not shown).

We also measured serum Ig levels after 2 and 4 weeks of treatment with TACI-Ig. The serum levels of IgM dropped 2.3-fold after 2 weeks of TACI-Ig treatment, but there was no significant drop in IgG compared to animals treated with Hu-Ig or PBS (Figure 1C). Similar data were obtained from animals treated with TACI-Ig for 4 weeks (data not shown). The longer half-life of IgG compared to IgM may be responsible for sustained levels of IgG in TACI-Ig-treated mice despite their reduced B cell numbers. Alternatively, B cells responsible for IgG production in naive mice (including the long-lived plasma cell pool) may be resistant to TACI-Ig in this treatment mode. In addition, an immune response to the human TACI-Ig protein may result in activation events that affect serum Ig. However, we have observed a similar effect on B cell development and Ig production in mice treated with murine TACI-Ig (data not shown) and in TACI-Ig TG mice (see below).

TG Mice Overexpressing TACI-Ig Have a Severe Depletion of Transitional T2 and Mature B Cell Populations and Reduced Levels of Circulating Ig

TG mice overexpressing TACI-Ig were produced using the V_H-E_μ promoter/enhancer to direct expression in lymphoid cells (Gross et al., 2000). Since normal mice mount an immune response to human TACI-Ig, we constructed TACI-Ig TG mice to study the effects of long-term exposure to the soluble TACI receptor in animals tolerant to the human protein. We identified expression of the transgene in serum from two founder mice (4687 and 4671) by Western blot analysis using polyclonal Abs to TACI (Figure 2A) and anti-human Fc Ab (data not shown). The two MW species identified in TG serum represent active proteolytic fragments of TACI-Ig. The levels of TACI soluble receptor were found to be 0.6 ± 0.075 mg/ml in mice from line 4687 (Figure 2B) detected by ELISA. Serum levels of both IgM and IgG were depressed in the TACI-Ig TG mice. There was an average 2.5-fold drop in serum IgM and a 5-fold drop in serum IgG levels measured in 20 TG mice from line 4687 (ages 9–23 weeks) compared to average values measure in 7 littermate controls of similar age (Figures 2C and 2D). Thus, chronic expression of TACI-Ig in the TG mice leads to a decrease in IgG levels that was not observed in normal adult mice treated for a short time with protein (Figure 1).

The total numbers of cells comprising the peripheral lymphoid compartments were determined in the TACI-Ig TG mice and littermate controls (Figure 2E). Total splenocyte numbers in adult TACI-Ig TG mice were 1.7-fold lower than splenocyte numbers in control mice (Figure 2E, $p = 0.0004$). In addition, there was a statistically significant decrease ($p = 0.0006$) in the numbers of B-1

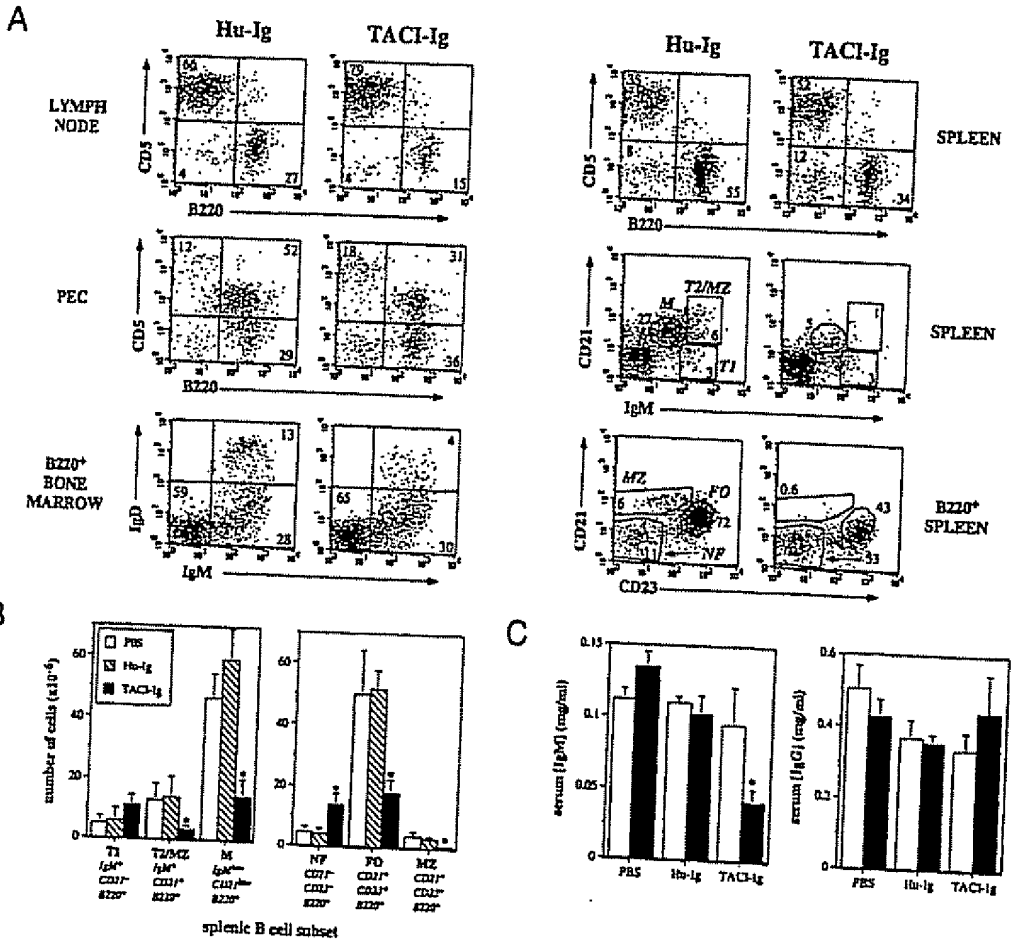


Figure 1. Mature B Cells and Serum IgM Levels Are Reduced in TACI-Ig-Treated Mice

Five BALB/c mice were treated three times/week for 14 days with PBS (data not shown), 100 μg of TACI-Ig, or Hu-Ig control protein. On the 14th day, thymus, mLN, spleen, BM, and PEC were collected, stained with fluorescently labeled mAbs to a variety of cell surface markers, and analyzed by flow cytometry.

(A) Representative profiles are shown for mLN, PEC (lymphocyte-gated), BM, and whole or B220+ spleen. PEC, mLN, and spleen were stained with anti-CD5 and anti-B220 mAbs to distinguish T and B cells. BM cells were stained with mAbs to IgM, IgD, and B220 and gated on B220+ lymphocytes. Splenic B cell subsets were identified as described in the text by simultaneously staining for CD21 and IgM to distinguish T1, T2/MZ, and M B cells, or for CD21, CD23, and B220 to identify NF, FO, and MZ B cells within the B220+ population.

(B) BALB/c mice were treated as described in (A) with PBS (open bars), Hu-Ig (hatched bars), or TACI-Ig (solid bars). Comparisons of the number of B cells in T1, T2/MZ, and M (left panel), and NF, FO, and MZ (right panel) for each group of mice are shown, and the cell surface markers used to define each subset are indicated. Error bars depict the standard deviation from the mean of each group; asterisks denote numbers whose p values (as calculated by Student's unpaired t test) were significantly different than the other two groups ($p < 0.05$).

(C) The levels of IgM (left panel) and IgG (right panel) in the serum of the mice described in (B) were assessed either before (open bars) or after (solid bars) 14 days of the indicated treatment.

and B-2 PEC in the TG mice (Figure 2E). However, the TACI-Ig TG mice have normal numbers of cells in the BM, thymus, and mLN compared to control mice (Figure 2E).

The thymus, mLN, spleen, BM, and PEC were isolated from ten TG mice and five non-TG. Cell populations were analyzed by FACS to characterize the effect of the TACI-Ig transgene on the developing immune system. Representative FACS analyses of these populations are shown in Figure 3A, and a detailed quantitative analysis of total cell numbers is shown in Figure 3B. The thymus of TACI-Ig TG mice contains normal percentages of immature and mature thymocytes defined by CD4 and CD8 expression, and the ratios of $CD4^+$ and $CD8^+$ T cells are normal in spleen, mLN, and thymus (data not shown). The average percentage of B-2 cells was reduced from 28.6 ± 2.9% to 3.9 ± 1.6% in the mLN ($CD19^+$), from 56.1 ± 6.2% to 19.9 ± 6.2% in the spleen ($B220^+CD5^-$), and from 37.2 ± 6.9% to 7.8 ± 3.1% in the peritoneum ($B220^+CD5^-$) (Figure 3A). There was also a loss of B-1 cells in the peritoneum, dropping from 14.5 ± 3.4% in non-TG mice to 4.3 ± 2.8% in mice expressing TACI-Ig, representing a 3.5-fold drop in the total number of

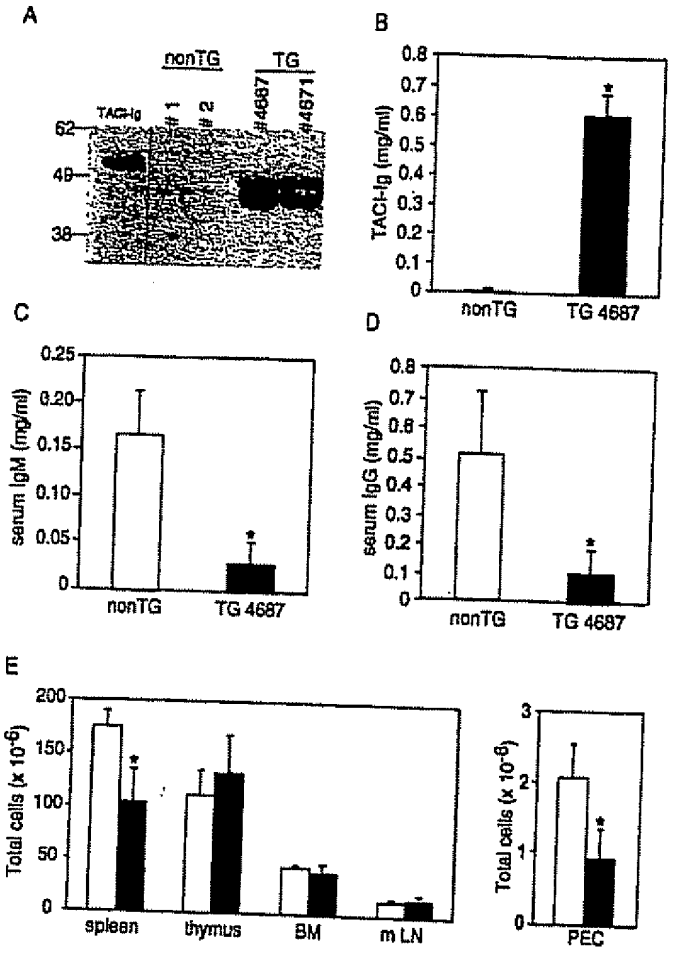


Figure 2. TACI-Ig TG Mice Have Reduced Numbers of Splenocytes, PEC, and Decreased Levels of Serum Ig

(A) 10 ng TACI-Ig (left lane) and 2.5 μl of serum from non-TG mice (#1 and #2) and two independent TACI-Ig TG founders (#4687 and #4671) were resolved on a NuPage MES gel (4%–12%), immunoblotted with TACI-specific rabbit polyclonal Abs, detected with HRP-conjugated anti-rabbit Ig, and developed using chemiluminescence. The predicted MW from the TACI-Ig TG construct is smaller than the MW of the TACI-Ig standard used in the Western blot. Partial proteolysis of TACI-Ig was observed in serum from both founders #4687 and #4671, resulting in two MW species approximately 46 and 44 kDa.

(B) TACI-Ig serum levels were determined by ELISA for offspring of TACI-Ig TG founder #4687. Values represent the average TACI-Ig concentration in the serum of four non-TG and four TACI-Ig TG mice (8–10 weeks of age). Error bars depict the standard deviation from the mean of each group; the asterisk indicates a statistically significant difference between the two groups ($p < 0.05$). The levels of (C) IgM and (D) IgG in serum from non-TG mice (open bars) and offspring of TACI-Ig TG founder #4687 (solid bars) were determined by ELISA. Values represent the average of 7 non-TG and 20 TACI-Ig TG mice, from 9–23 weeks of age; statistical significance of the data was determined as in (B).

(E) Cell suspensions from spleen, thymus, BM, mLN, and PEC were prepared from non-TG and TACI-Ig TG mice and numbers of trypan blue-excluding live cells were counted. The average total cell number in each organ/compartment of five non-TG (open bars) and ten TACI-Ig mice (solid bars) is shown.

B-1 cells (Figure 3). This suggests that the B-1 cells may be dependent on BLyS and/or APRIL for their survival or development (see Discussion).

There is a clear loss of mature $IgM^{low}IgD^{+}$ (Fraction I), as well as the less mature Fractions II ($IgM^{+}IgD^{+}$) and III ($IgM^{+}IgD^{low}$) (Hardy et al., 1982) B cells in the spleen of non-TG versus TACI-Ig TG mice (Figure 3). There is also a specific arrest in the development of both T2 and MZ B cells ($CD21^{+}$ IgM^{+}), decreasing from 11.7 ± 3.2% in control mice to 1.6 ± 0.6% in mice expressing TACI-Ig, whereas the T1 transitional B cells remain unaffected (Figure 3A). It should be noted that "Fraction III" (Hardy et al., 1982) and the T1 cell population are not equivalent, since Fraction III actually consists of a mixture of NF/T1, MZ, some B-1, and other activated B cells. Thus, there is no discrepancy in the observation that the number of Fraction III B cells drops dramatically in the TACI-Ig TG mice, while the numbers of T1 B cells are comparable to those in the non-TG controls (Figure 3B). There is also a distinct block in the development of MZ B cells in the TACI-Ig TG mice (Figure 3A). Given the compelling similarities between B-1 and MZ B cells (Martin and Kearney, 2000a), our data suggest that a common set of factors, which likely include BLyS and/or APRIL, are important for the development of both these B cell populations.

There is also a dramatic decrease in the T2 B cell population and mature FO B cell populations, whereas the numbers of T1 B cells are relatively unaffected in TACI-Ig TG mice (Figure 3B). There is an increase in $CD21^{hi}CD23^{+}$ B cells in the spleen of TACI-Ig TG mice (Figure 3B). This may represent a novel transitional population or alternatively a downmodulation of CD21 on the surface of the FO B cell population. BM from TACI-Ig TG mice contains slightly lower numbers of $IgM^{+}IgD^{-}$ immature B cells (Figure 3B). Remarkably, despite the substantial loss of mature B cells, there is only a subtle, but significant, decrease in the numbers of $CD4^{+}$ and $CD8^{+}$ T cells in the spleen and mLN of TACI-Ig TG mice (Figure 3B; data not shown).

TACI-Ig Inhibits the Progression of Disease and Autoantibody Titers in a Mouse Model of CIA

There are elevated levels of BLyS in the serum of patients with RA (Cheema et al., 2001) and SLE, correlating with elevated levels of autoreactive Abs (Zhang et al., 2001). Therefore, BLyS represents an appropriate target for intervention in autoimmune diseases characterized

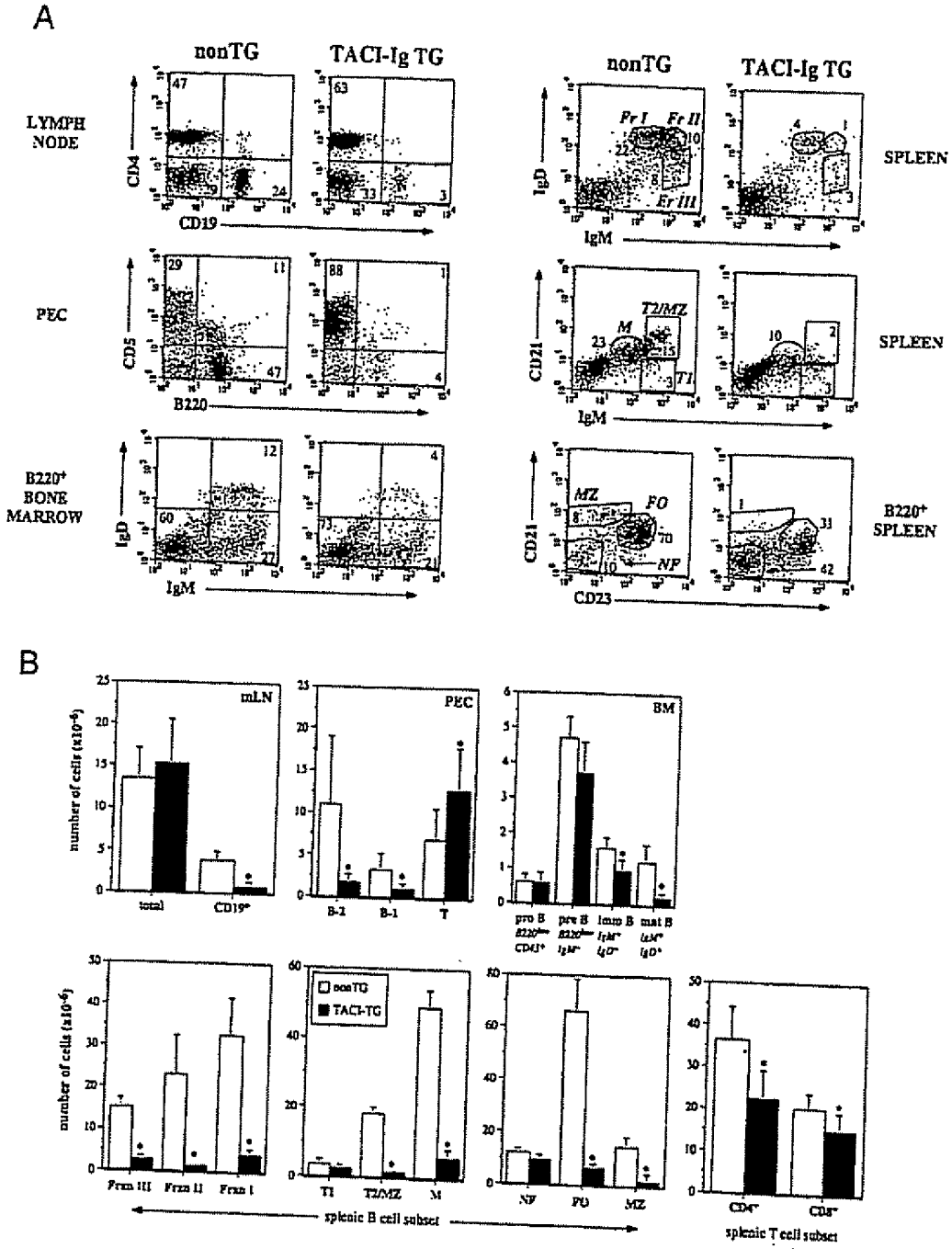
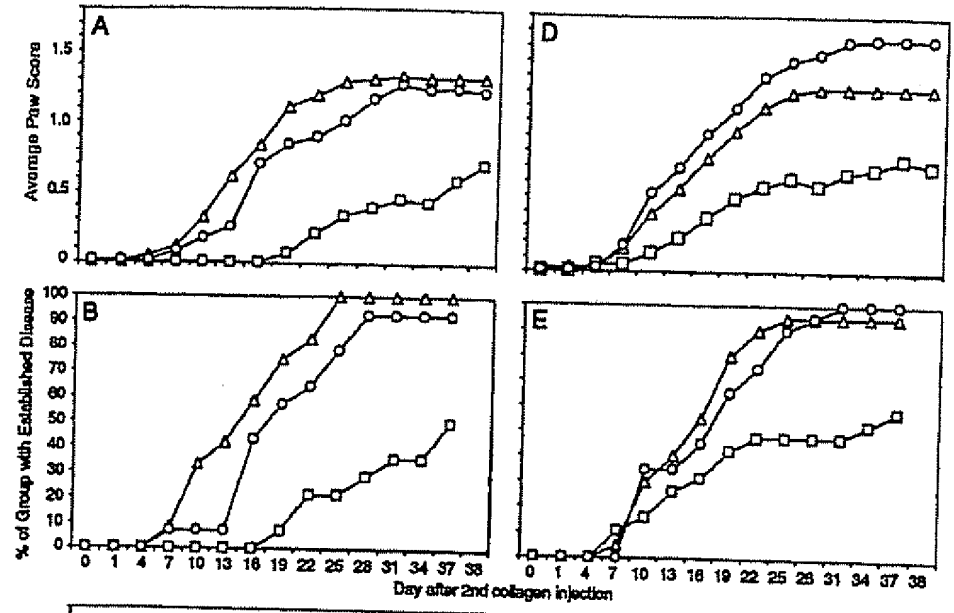
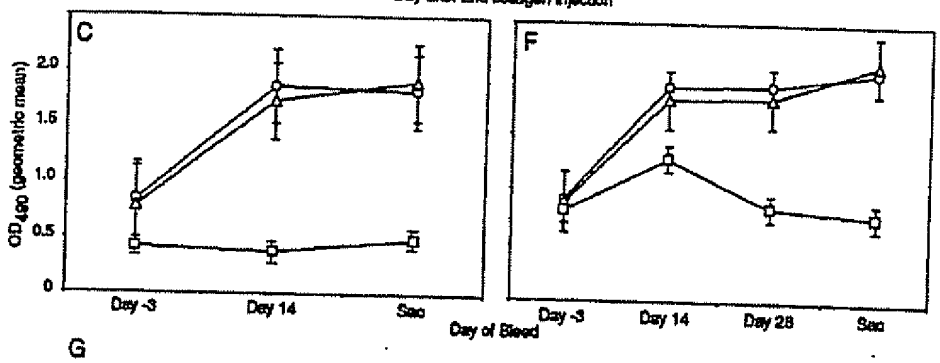


Figure 3. The B Cell Compartment in TACI-Ig TG Mice Is Dramatically Altered Relative to That of Non-TG Mice

(A) Cell suspensions from non-TG (left columns) or TACI-Ig TG (right columns) BM, mLN, spleen, and PEC were stained as described in Figure 1 and analyzed by flow cytometry, and splenic B cell subsets were identified as described in Figure 1. Splenocytes were also costained with anti-IgD and anti-IgM Abs to distinguish Fractions III (*FrIII*), II (*FrII*), and I (*FrI*) as described in the text.

(B) The average number of the following cell types from five non-TG (open bars) or ten TACI-Ig TG (solid bars) mice are plotted (upper panels): total or CD19$^+$ B cells in the mLN; B-2, B-1, and T cells in the PEC; or pro B (B220^{low}CD43$^+$), pre B (B220^{low}CD43$^-$IgM$^-$), immature B, and mature recirculating B cells in the BM. The average number of each splenic B cell subset, as well as the total number of CD4$^+$ and CD8$^+$ splenic T cells are also plotted (lower panels). Error bars represent the standard deviation from the mean; asterisks denote statistically significant differences ($p < 0.05$).





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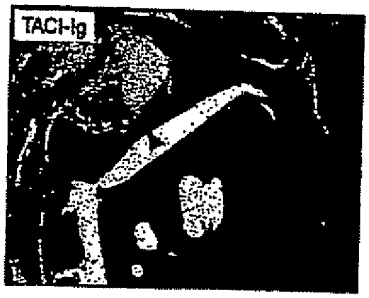

Treatment	Paw Score	Joint Score
□ TACI-Ig	3.3 +/- 4.1	0.5 +/- 0.6
△ Hu-Ig	6.2 +/- 4.1	1.3 +/- 0.9
○ PBS	11.3 +/- 4.6	2.2 +/- 1.1

by elevated levels of autoantibodies that contribute to disease pathology. The ability of TACI-Ig to suppress the development of Abs to foreign antigens indicates that it might be useful in suppressing the development of autoreactive Abs and disease in the CIA model of RA.

We treated mice with TACI-Ig, Hu-Ig or PBS three times a week, i.p., for 3 weeks. In the prevention mode, treatment was initiated 7 days prior (day −7) to the second collagen injection (day 0), when there are already increased serum titers of anti-collagen Abs and few overt signs of disease (Figures 4A–4C). Qualitative clinical scores of disease were assessed daily for all four paws (scale 0–3). The average paw score and the percentage of animals with established disease were reported for each group. In addition, serum samples were tested for circulating anti-collagen Abs at day −3, day 14, and day of sacrifice (Figure 4C).

Prophylactic administration of TACI-Ig delayed the onset and lowered the severity of inflammation in the TACI-Ig treatment group (Figure 4A). No disease was observed until after day 18, 4 days after dosing was stopped. In addition, TACI-Ig decreased the percentage of animals that developed established disease compared to mice treated with Hu-Ig or PBS (Figure 4B). TACI-Ig suppressed the development of anti-collagen Abs on day 14 (3 weeks after treatment was initiated) and on day of sacrifice, compared with the Hu-Ig and PBS control groups (Figure 4C).

Administration of TACI-Ig 7 days after the second dose of collagen more closely represents a therapeutic mode of treatment. At this time, anti-collagen Abs are elevated, and inflammation was present in an average of 52% of the animals (data not shown). Administration of TACI-Ig at this stage inhibited the progression of disease, reflected in lower average paw scores (decreased severity of inflammation) (Figure 4D) and in the decreased percentage of animals with established disease (Figure 4E). The serum Ab titers to collagen were slightly decreased in the TACI-Ig treatment group at day 14, 1 week after the start of treatment, and decreased further in serum samples taken on day 28 and day of sacrifice (Figure 4F). Immunohistochemical staining of spleen cryosections revealed that splenic architecture of TACI-Ig treated mice was normal but contained fewer FO B cells (data not shown).

Histological assessment of arthritis in interphalangeal joints of paw sections revealed that mice receiving TACI-Ig had lower average paw and joint scores (3.3 and 0.5, respectively) compared to the PBS-treated mice (11.3 and 2.2) or Hu-Ig treated mice (6.2 and 1.3) (Figure 4G). Overall, these results indicate a reduction in disease in mice receiving TACI-Ig. Specifically, the articular cartilage in the TACI-Ig treated group generally was undamaged, and there was less inflammation and hypertrophy of the synovium (Figure 4G). In addition, the TACI-Ig-treated group had fewer inflammatory cells in the soft tissues surrounding the joints (Figure 4G).

The Phenotype of BLyS$^{-/-}$ Mice Demonstrates that BLyS Is Required for the Development of B-2 B Cells but Not B-1 Cells

To elucidate the involvement of BLyS in B cell differentiation and homeostasis, we produced *BLyS*-deficient mice by targeted gene disruption. The targeting strategy was designed to delete sequences encoding amino acids 2–86 of *BLyS*, encompassing the cytoplasmic tail and transmembrane domain (Figure 5A). *BLyS*$^{-/-}$ mutant offspring were found to be present at the expected Mendelian ratio (23%: 54%: 23%, respectively; n = 75). *BLyS*$^{-/-}$ animals were viable, appeared outwardly normal, and showed no obvious differences in size, behavior or reproductive ability. No *BLyS* expression was detected in adult lung or spleen isolated from *BLyS*$^{-/-}$ mice (Figure 5C; data not shown), demonstrating that a null allele had indeed been generated.

The average levels of serum IgG and IgM were reduced 4.6- and 3.9-fold, respectively, in *BLyS*$^{-/-}$ mice compared to Ig levels in WT mice (Figure 5D). Cell populations in the spleen, thymus, PEC, mLN, and BM were characterized by FACS in ten *BLyS*$^{-/-}$ mice and nine age-matched control mice, ranging from 6 to 16 weeks of age. Total cell numbers in different lymphoid compartments were calculated (Table 1) and representative FACS of specific cell subsets are shown (Figure 6). The total numbers of cells isolated from thymus, BM, and PEC from *BLyS*$^{-/-}$ mice were not significantly different than numbers obtained from wild-type mice (Table 1). However, the total numbers of cells found in spleen and LNs of *BLyS*$^{-/-}$ mice were reduced compared to WT mice, from 199.7 ± 36.6 ($\times 10^{-6}$) to 75.5 ± 24.0 ($\times 10^{-6}$) in the spleen and from 28.7 ± 3.2 ($\times 10^{-6}$) to 9.9 ± 0.5 ($\times 10^{-6}$) in the mLN.

The *BLyS*$^{-/-}$ mice contained normal thymocyte populations defined by CD4 and CD8 (data not shown) and normal numbers of CD3$^+$ peripheral T cells in the spleen (Table 1) and mLN (data not shown), indicating that *BLyS*$^{-/-}$ T cells can differentiate and populate the periphery. However, it remains to be determined whether these

Figure 4. TACI-Ig Treatment Before or during the Onset of CIA Prevents the Production of Anti-Collagen Abs and Dramatically Reduces the Severity of the Disease

(A–F) Mice were immunized with type II chick collagen emulsified in CFA on day −21 and IFA on day 0. Treatment was initiated on day −7 (A, B, and C) or on day +7 (D, E, and F). Fifteen mice per group received PBS (circles) or 100 μg of either Hu-Ig (triangles) or TACI-Ig (squares) i.p. three times/week for 3 weeks. Arthritic lesions for each paw were graded daily using a scoring system of 0–3 (see Experimental Procedures), and mice were identified as diseased if the score of one paw was at least 2. The average paw score was calculated for each group (A and D), and the average number of animals with disease was determined (B and E). (C and F) Collagen-specific Abs were measured in serum samples drawn on the days indicated. The results were reported as geometric mean and standard error of the OD_{405} values from ELISAs detecting collagen-specific Abs.

(G) Histopathology of the joints from four paws was assessed on six mice from each treatment group, sacrificed 7 days after being diagnosed with disease. Representative sections stained with H&E are shown from the PBS-, Hu-Ig-, and TACI-Ig-treated groups, as indicated. Arrowheads indicate the surface of the articular cartilage. Pathology scores of 0–4 were assigned for paws and digits (see Experimental Procedures) of six mice in each group (table); mean scores and the standard deviation from the mean are reported.

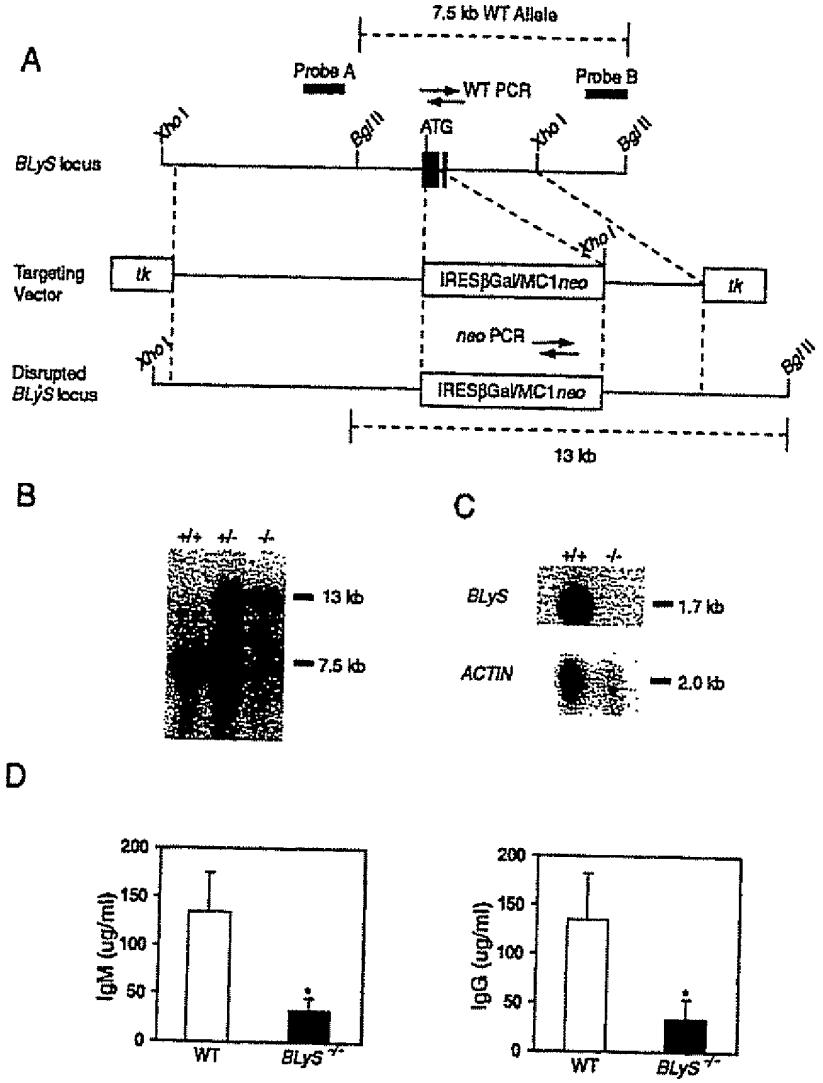

Figure 5. *BLyS*-Deficient Mice Generated by Gene Disruption Have Reduced Levels of Circulating Ig

(A) A partial restriction endonuclease map of the wild-type mouse *BLyS* locus (top), structure of the *BLyS* targeting vector (middle), and resulting homologous recombinant allele (bottom) are shown, in which sequences encoding amino acid residues 2–80 were deleted and replaced with a *lacZ* gene and *neo* selectable marker (IRESβGAL/MC1*neo*). Exons 1 and 2 of *BLyS* are indicated by black boxes, and exon 3 is not shown. ES cells and animals were genotyped by genomic Southern blotting using probes A and B (horizontal black bars) to confirm the recombinant 5′ and 3′ junctions, respectively. The expected sizes of BglII-digested bands (horizontal dashed lines) representing the wild-type (WT) allele and *BLyS* null allele was detected by Southern hybridization with 3′ probe B. PCR primer pairs used to detect the WT and *neo* alleles are indicated by opposing black arrows and were used to genotype most of the progeny.

(B) Genomic Southern blot analysis using 3′ probe B of BglII-digested tail DNA from $BLyS^{+/+}$ (+/+) and $BLyS^{-/-}$ (−/−) mice derived from breeding $BLyS^{+/-}$ (+/−) mice.

(C) Northern blot analysis of poly A$^+$ RNA isolated from the lungs (2 μg/lane) of $BLyS^{+/+}$ (+/+) and $BLyS^{-/-}$ (−/−) mice. Hybridization was performed with a *BLyS* exon 3–specific radioactive probe (upper panel). As a control for equal RNA loading, blots were stripped and rehybridized with a mouse actin probe (lower panel).

(D) The levels of IgM (left panel) and IgG (right panel) in the serum of WT mice (open bars) and $BLyS^{-/-}$ (solid bars) were assessed by ELISA. Values represent the average of five WT and nine $BLyS^{-/-}$ mice, from 10–14 weeks of age; asterisks denote statistically significant differences ($p < 0.05$).

are normal functional T cells. The expression of TACI on the surface of activated T cells (Bram et al., 2000) potentially implicates the ligands for TACI in the processes of T cell stimulation and/or differentiation into effector T cells. The numbers of CD44$^+$CD62L$^-$ memory/effector T cells were reduced from 13.6 ± 5.6 (× 10^{-6}) in WT mice to 6.6 ± 2.2 (× 10^{-6}) in $BLyS^{-/-}$ mice (Table 1). This represents a small but statistically significant

Table 1. Peripheral B Cell Numbers Are Dramatically Reduced in *BLyS*$^{-/-}$ Mice

	Cell Counts ($\times 10^{-6}$)[a]		
	WT	*BLyS*$^{-/-}$	*p* value[b]
Thymus	161.1 ± 100.7	166.3 ± 43.7	0.94
Spleen	199.7 ± 38.6	75.5 ± 24.0	<0.0001
mLN	28.7 ± 3.2	9.9 ± 0.5	0.0002
BM[c]	9.6 ± 2.6	9.8 ± 3.1	0.87
PEC	2.2 ± 1.2	1.5 ± 0.4	0.19
Spleen B ($B220^+$)[d]			
T1	9.0 ± 4.6	10.0 ± 4.7	0.58
T2/MZ	19.2 ± 14.5	0.6 ± 0.6	0.0002
Mature	68.0 ± 18.7	3.4 ± 1.2	<0.0001
NF	12.4 ± 3.3	16.8 ± 7.1	0.11
FO	81.7 ± 28.2	0.5 ± 0.2	<0.0001
MZ	11.6 ± 6.7	0.5 ± 0.3	<0.0001
Spleen T ($CD3^+$)[d]			
All T	77.7 ± 12.6	48.8 ± 17.0	0.082
Naive T	45.7 ± 17.0	35.5 ± 13.7	0.0046
Memory T	13.6 ± 5.6	6.6 ± 2.2	0.0018
PEC			
B-2	0.18 ± 0.15	0.04 ± 0.03	0.07
B-1	0.17 ± 0.17	0.07 ± 0.04	0.18
BM ($B220^+$)			
IgM^-D^-	4.5 ± 0.7	5.2 ± 1.5	0.26
Immature IgM^+D^-	2.3 ± 0.7	2.4 ± 0.7	0.75
Mature IgM^-D^+	0.8 ± 0.2	0.2 ± 0.05	<0.0001

[a] To obtain the number of cells in each subpopulation, the total number of trypan blue-excluding cells was multiplied by the percentage of cells in each subset, as determined by FACS analysis (Figure 6). Values represent the mean ($\times 10^{-6}$) and standard deviation from the mean of data from 7–9 WT and 7–10 *BLyS*$^{-/-}$ mice.
[b] *p* values were calculated using Student's unpaired *t*-test; values deemed statistically significant ($p < 0.05$) are in boldface.
[c] BM cell counts represent the number of cells isolated from one hindleg femur.
[d] B and T cell subsets are defined as described in Figure 6.

($p = 0.0018$) decrease in effector T cells in mice lacking *BLyS*.

B cell development is blocked at the transitional T1 stage ($CD21^-IgM^+$) in *BLyS*$^{-/-}$ mice such that virtually no T2, MZ, or FO B cells are present (Table 1; Figure 6). NF B cells are present in normal numbers in the spleens of *BLyS*$^{-/-}$ mice and represent the majority of B cells in the spleen (Table 1). Immunohistochemical staining of spleen cryosections from 4 *BLyS*$^{-/-}$ mice revealed normal splenic architecture in *BLyS*$^{-/-}$ mice. Metallophilic macrophages were normal, but there was an increase in $CD3^+$ T cells and reduction of FO B cells (data not shown).

Analysis of the BM revealed that *BLyS*$^{-/-}$ mice have normal numbers of pre/pro and immature B cells but reduced numbers of mature recirculating B cells (Table 1; Figure 6). We compared the percentages and total numbers of B-1 cells in the peritoneum of WT and *BLyS*$^{-/-}$ mice (Figure 6; Table 1). The average percentage of B-1 cells defined as $B220^{low}CD5^{low}$ were not dramatically different in *BLyS*$^{-/-}$ mice compared to percentages in WT mice (32.6 ± 9.1% and 28.0 ± 9.5%, respectively). The total numbers of B-1 B cells in the peritoneum of *BLyS*$^{-/-}$ mice were not significantly different from WT mice, while the peritoneal B-2 cell population in these mice is severely reduced (Table 1; Figure 6).

Discussion

The growing number of TNF ligands and receptors with multiple partners has added to the complexity of dissecting the biological effects mediated by this family of proteins. Exemplifying this complexity is the association of BLyS and APRIL with two known receptors, TACI and BCMA (Gross et al., 2000; Marsters et al., 2000) and recent evidence for a potential new receptor for these molecules (our unpublished data). Both BLyS and APRIL are capable of stimulating B cells in vitro (Moore et al., 1999; Schneider et al., 1999; Yu et al., 2000), and BLyS can expand B cell populations in vivo (Gross et al., 2000; Mackay et al., 1999), whereas APRIL has been shown to costimulate T cells (Yu et al., 2000) and to enhance the survival of some tumors (Hahne et al., 1998). Several experiments have demonstrated an association between elevated levels of circulating BLyS and autoimmune disease, including SLE in mice (Gross et al., 2000) and humans (Zhang et al., 2001) and RA in humans (Cheema et al., 2000), whereas APRIL has been implicated in cancer (Ware, 2000). We report here experiments that further characterize the biology associated with this family of proteins. We have used TACI-Ig, a protein capable of neutralizing both BLyS and APRIL, to understand the role of these ligands in normal and diseased mice. We have also produced *BLyS*-deficient mice to elucidate the biology of this ligand and its role in the developing immune system.

The introduction of TACI-Ig to preimmune mice achieved either by treatment with soluble protein or by overexpression of TACI-Ig as a transgene, led to a disruption in B cell development without substantially affecting other cell populations (Figures 1–3). This suggests that either BLyS or APRIL is made constitutively

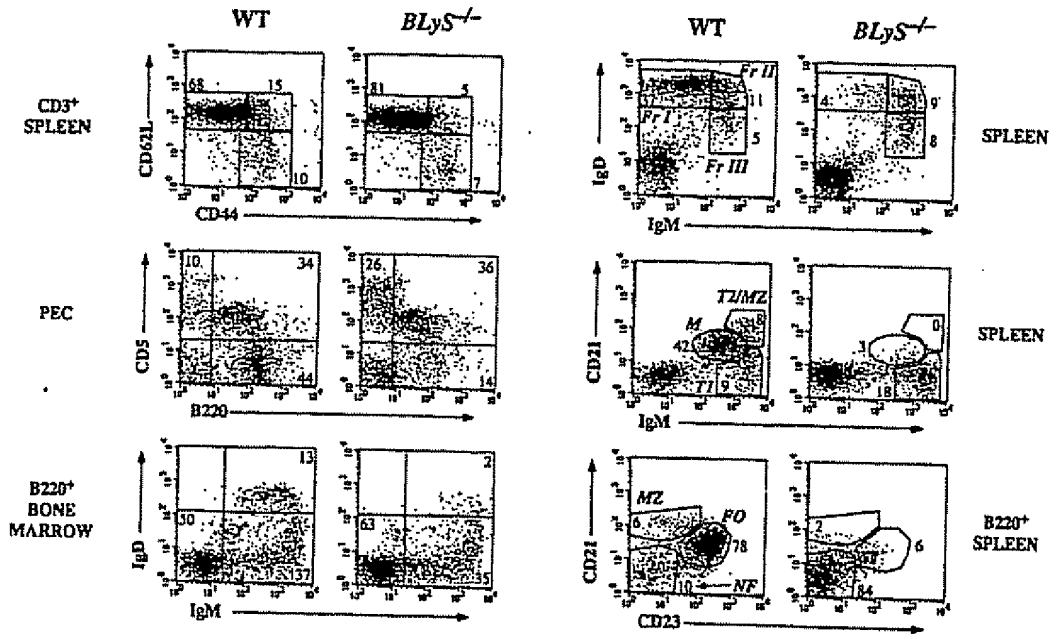

Figure 6. *BLyS*-Deficient Mice Lack Mature Splenic B Cells but Have Apparently Normal Populations of Peritoneal B-1 Cells and Immature BM B Cells

Splenocytes, PEC, and BM cells from ten 6- to 16-week-old *BLyS*$^{-/-}$ mice and nine age-matched wild-type (WT) control mice were stained with mAbs to the cell surface molecules indicated and analyzed by flow cytometry as described in Figures 1 and 3. Splenocytes were also stained with mAbs to CD44, CD62L, and CD3 and gated on CD3$^+$ lymphocytes to determine the relative distribution of naive (CD44$^-$CD62L$^+$), "activated"(CD44$^+$CD62L$^+$), and memory (CD44$^+$CD62L$^-$) T cells. Representative plots for whole lymphocyte-gated, B220$^-$, and CD3$^+$ spleen, lymphocyte-gated PEC, and B220$^-$ BM are shown. The percentages of cells in each quadrant or region are indicated.

and is required for the development and homeostasis of the peripheral B cell pool.

Characterization of splenic B cells in both TACI-Ig-treated or TG mice revealed a specific arrest in B cell development at the immature transitional T1 stage (Figures 1 and 3). The number of T1 cells in these mice was unaffected by TACI-Ig but there was a dramatic loss of T2, mature, and MZ B cells in the spleen. These results suggest that the factors neutralized by TACI-Ig are required to drive B cell differentiation at the T1 stage or are required to sustain mature B cell populations. The unique population of splenic CD21^{low}CD23$^+$ B cells identified in TACI-Ig TG mice may represent a novel transitional B cell population. Alternatively, CD21 could be downmodulated as a result of exposure to high levels of TACI-Ig in the TG mice. Analysis of the lymphoid compartment of *BLyS*$^{-/-}$ mice demonstrates the requirement for this ligand in the development of mature B cell populations. The block in B cell differentiation past the T1 stage observed in TACI-Ig TG mice is even more pronounced in spleens from *BLyS*$^{-/-}$ animals. The total number of splenic T1 cells is the same in *BLyS*$^{-/-}$ and WT mice, but the mature, T2, and MZ B cells are completely absent in spleens of mutant mice (Figure 6; Table 1). The arrest in B cell development is restricted to the peripheral mature and immature B cell populations. Normal B cell precursor populations (pro- and pre-B cells) are present in BM isolated from TACI-Ig treated mice and TACI-Ig TG mice (Figure 3). This observation, and the lack of perturbation of these cells in the BM of *BLyS*$^{-/-}$ mice, suggests that B cells will repopulate the periphery after TACI-Ig is cleared from the animal.

Recent experiments have shown that BLyS can act as a survival factor for B cells. One group demonstrated that BLyS is sufficient to prolong the survival of naive resting B cells in vitro by attenuating apoptosis, independent of antigen stimulation (Do et al., 2000). Another group showed that BLyS preferentially induces the survival in vitro of splenic immature T2 B cells but not T1 or mature cells (Batten et al., 2000). Our data support a role for BLyS in the differentiation from T1 to T2 but we cannot rule out a role for BLyS as a survival factor for the T2 population.

A functional BCR is absolutely required for the development of B cells, but a variety of other receptors and signals from the microenvironment also provide essential cues necessary for proper B cell development (Loder et al., 1999). B cell maturation in mice deficient for the BCR signaling element *Igα* (*mb1Δc/Δc*) is arrested somewhat before or just at the T1 stage in the spleen (Torres et al., 1996). The T1 population in these mice is 20% of normal, indicating the need for BCR signaling during the differentiation from immature BM-derived B cells to the transitional T1 stage. B cells from *Syk*$^{-/-}$ mice migrate from the BM to the spleen but do not enter into the primary follicle and instead remain in the red pulp and outer PALS (Turner et al., 1995). *Pyk*-2-deficient mice do not develop MZ B cells but have normal B-1

and FO B-2 cells (Guinamard et al., 2000). Taken together, these studies demonstrate the key roles played by specific molecules during B cell development. The phenotype of *BLyS*$^{-/-}$ mice indicates that this factor is important for the T1 to T2 transition and may represent one of the factors supplied by the microenvironment supporting these B cell populations.

TACI-Ig treatment has little effect on T lineage cells or myeloid cell populations in the spleen, mLN, and PEC (Figure 1; data not shown). The high levels of TACI-Ig produced in the TG mice results in a small reduction in the numbers of CD4$^+$ and CD8$^+$ T cells in the spleen (Figure 3B). We can conclude from our findings that neither APRIL nor BLyS plays a significant role in development of naive T cells in a preimmune state.

The biological effect of APRIL in vitro suggests a role for this molecule in T cell costimulation (Yu et al., 2000). Analysis of the mature T cell populations in *BLyS*$^{-/-}$ mice indicates a potential role for BLyS in the development of memory T cells in the spleen (Table 1). Whether the subtle drop in T cell numbers in the TACI-Ig TG or the drop in effector T cells in *BLyS*$^{-/-}$ mice is the result of a direct effect of TACI-Ig on the T cells themselves, or of an indirect effect stemming from the paucity of B cells, is not yet clear. Studies on B cell-deficient μMT mice clearly demonstrate a role for B cells in the generation of a T cell response to parasitic and viral infection (Langhorne et al., 1998; Homann et al., 1998). More experiments are needed to examine the production of effector T cells during antigenic challenge and T cell selection in *BLyS*-deficient mice or during TACI-Ig treatment of normal mice.

The role of BLyS in B cell activation and humoral immunity has suggested a utility for TACI-Ig in the treatment of autoimmune diseases associated with pathogenic autoantibodies. Here we show that TACI-Ig can inhibit the incidence of disease in a model of CIA either when administered before onset of disease (7 days prior to the second antigen challenge) or when 52% of the mice have overt signs of inflammation (7 days after the challenge). In both delivery modes, TACI-Ig suppresses the production of anti-collagen Abs that correlate with inhibition of disease progression. Evidence supporting a role for B cells in the development of CIA has been shown in B cell-deficient μMT mice. These mice do not develop CIA, but their anti-collagen T cell response is normal (Svensson et al., 1998).

Studies have shown that TACI-Ig treatment can inhibit the production of Abs to T cell-dependent antigens, GC formation, high-affinity IgG production, and formation of low-affinity IgM (Yan et al., 2000). In the CIA model, TACI-Ig was delivered 14 to 21 days after the first dose of priming antigen, after GCs have formed. We can postulate several mechanisms by which TACI-Ig suppresses Ab formation. TACI-Ig may act by inhibiting the survival of mature B cells, reducing the pool of cells that can develop into Ab-forming cells. Similarly, TACI-Ig could act directly by inhibiting the differentiation of B cells into Ab-producing plasma cells. TACI-Ig may act by inhibiting factors required for the maintenance of GCs (suggested by our experiments) or completely inhibiting GC formation. Regardless of the mechanism, the data taken together indicate that TACI-Ig is a potent inhibitor of mature B cells and the production of antigen-specific Abs and therefore may be useful in suppressing pathogenic autoantibodies in disease.

The mature long-lived pool of B cells is composed of the B-1 B cell subset, MZ B cells, and mature B-2 B cells that recirculate among the follicles. The long-lived pool of B-2 B cells is derived from NF B cells that mature through the transitional stage in the spleen. Alternatively, mature B cells can migrate to more static compartments including the MZ B cells or peritoneal cavity (B-1 B cells). The origin of B-1 cells is less clearly understood, but this population may derive directly from fetal or adult precursor populations (Wortis and Berland, 2001). Analysis of the PEC population isolated from *BLyS*$^{-/-}$ mice revealed the most striking difference between the *BLyS*$^{-/-}$ mutant mice and TACI-Ig -TG and -treated mice. The PEC from *BLyS*$^{-/-}$ mice contained very few B-2 B cells but had normal levels of B-1 B cells compared to WT mice. We have shown previously that mice overexpressing BLyS as a transgene develop elevated B-1 cells in the spleen (Gross et al., 2000). The lack of B-1 cells in mice overexpressing TACI-Ig or in mice treated with TACI-Ig suggests that another ligand(s), possibly APRIL, acts in synergy with BLyS to modulate the development of this B cell population and may compensate for BLyS in the development of B-1 cells in the mutant mice.

These experiments indicate that BLyS plays an important role in the development of conventional B-2 cells but not in the development of peritoneal B-1 cells. By contrast, TACI-Ig clearly alters the development of both B-2 and B-1 B cells (Figures 1 and 3). We have shown that TACI-Ig treatment can inhibit symptoms of disease in a mouse model of SLE (Gross et al., 2000). Here we demonstrate the ability of TACI-Ig to suppress disease symptoms and the production of pathogenic Abs in CIA. In normal mice, we show that TACI-Ig acts as an immunosuppressive, regulating predominantly B cells without dramatically affecting other lymphoid or myeloid cell populations. Taken together, the ability of TACI-Ig to influence the survival of B-2, B-1, and MZ populations and to regulate antigen-specific Ab production in the CIA model underscores the therapeutic utility of this molecule in the treatment of autoimmune disease.

Experimental Procedures

Generation of Soluble TACI-Ig and Treatment of Normal Mice

The TACI-Ig protein was generated by fusing the signal sequence derived from human tissue plasminogen activator, the extracellular domain of human TACI (amino acids 1–154) (von Bulow and Bram, 1997), and a mutated Fc region (Fc4) from the human IgG1 heavy chain. To create Fc4, amino acid substitutions L234A, L235E, and G237A were introduced into IgG1 Fc to reduce binding to FcγRI, FcγRIIa, and FcγRIII (Duncan et al., 1988; Wines et al., 2000; Sondermann et al., 2000), and substitutions A330S and P331S were introduced to reduce C1q-mediated complement fixation (Tao et al., 1993; Canfield and Morrison, 1991). Protein was purified from supernatants derived from CHO DG44 cells expressing the TACI-Ig by Protein A chromotagraphy. BALB/c mice (Charles River Laboratories, Wilmington, MA) were treated via i.p. injection with 100 μg of TACI-Ig, Hu-Ig (the Fc portion of human IgG1 produced as described for TACI-Ig), or PBS three times/week for 14 days.

VλEμ-TACI-Ig TG Mice

A PCR fragment containing the TACI-Ig construct described above was inserted into the expression vector pKFO24 (Gross et al., 2000)

containing the $V_H/E\mu$ promoter/enhancer. Fertilized ova from matings of B6C3F1Tac mice were microinjected with the transgene insert and implanted into pseudopregnant females. Founders were identified by PCR on genomic tail DNA, and TG lines were established by breeding founders with C57BL/6Tac mice (Taconic, Germantown, NY).

BLyS$^{-/-}$ Mice

Heterozygous knockout mice were generated at Lexicon Genetics Incorporated (The Woodlands, TX), essentially as previously described (Wattler et al., 1999; Matalon et al., 2000). *BLyS* genomic clones were identified in a λKOS genomic library derived from mouse strain 129S5 (129/SvEvBrd). A 10.5 kb genomic clone was used to create a replacement-type targeting vector to delete a 334 bp sequence beginning with codon 2 of exon 1 and extending through the first 19 bp of intron 1 containing the cytoplasmic and transmembrane domains of BLyS (Figure 5). This deleted region was replaced by an IRESβGal-PolyA/MC1neo-PolyA selectable marker cassette that contains termination codons in all three reading frames, an internal ribosome entry sequence derived from encephalomyocarditis virus fused in-frame to the *lacZ* gene, a polyA signal, followed by a *neo*^{r} selectable marker (Thomas and Capecchi, 1987), and a second pA signal. The targeting vector contained two copies of the negative selectable marker *HSV-tk*, one flanking each side of the genomic insert. The linearized targeting vector was electroporated into 129S5-derived Lex-1 ES cells and subjected to positive/negative selection with G418 and fialouridine. A total of 22 (11%) targeted clones were identified by Southern blotting (Figure 5). Chimeras were generated by injection of targeted ES cells into C57BL/6-Tyr^{c-brd} blastocysts and transplantation into pseudopregnant females. Progeny were genotyped by Southern blotting employing probe B (Figure 5) or separate PCR reactions detecting the wild-type and *neo* alleles. Probe B was generated by PCR amplification of a 608 bp product from genomic clone pKOS/ZG7/66, using the following primers: ZC37420, 5′-CCATCCAGAATGCTCTACTG-3′ and ZC37422, 5′-CAGCCTTGC TTGGCTGCAC-3′. PCR genotyping was performed with the primer pairs: ZG37424 (sense wild-type *BLyS*), 5′-CTGCCACCACCGTGC CTCTGTT-3′ and ZG37457 (antisense wild-type *BLyS*), 5′-CCGCGG CGGCTGGTGTT-3′ for a 268 bp amplicon; ZG23131 (sense *neo*), 5′-GGGCGCCCGGTTCTTTTTGTC-3′ and ZG23133 (antisense *neo*), 5′-TTCGCCGCCAAGCTCTTCAGC-3′ for a 579 bp amplicon.

CIA Model

Male DBA/1 mice (Jackson Laboratory, Bar Harbor, ME) were injected with an emulsion of chick collagen type II (Chrondrex, LLC) in Arthrogen-CIA Adjuvant (Complete Freund's adjuvant [CFA] containing 1 mg/ml *Mycobacterium tuberculosis*, H37RA, Difco) on Day −21 s.c. at the base of the tail. On Day 0, the mice were given a second immunization of collagen emulsified in Incomplete Freund's adjuvant (IFA). All mice were assessed daily beginning on Day 0 by assigning a clinical score to each of their paws, based on the severity of edema and erythema. Arthritic lesions for each paw were graded on a score of 0–3, where 0 = normal, 0.5 = only toe(s) involved, 1 = mild edema and erythema in paw, 2 = moderate edema and erythema in paw, and 3 = severe edema and erythema in paw. The arthritic score for each animal each day was the sum of the clinical scores for all four paws. Animals were euthanized after 7 consecutive days of a score of 2 or more in any one paw. At necropsy, serum, inguinal, and mLN, and paws were collected. Paw histology was assessed formalin-fixed sections stained with H&E using the following scoring system: 0 = normal synovial membrane (2–3 cells thick), smooth articular surfaces; 1 = synovial membrane hypertrophy, cellular infiltrate into synovial space; 2 = grade 1, plus synovial membrane hypertrophy and superficial cartilage erosion; 3 = grade 2, plus major erosion of the cartilage and subchondral bone; and 4 = loss of joint integrity through erosion with massive cellular infiltrate. Each paw was given a score of 0–4, determined by the maximal joint score for that paw.

Northern Blot Analysis

RNA was isolated using the RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, with the exception that a DNase I treatment at 22°C for 15 min was included between RW1 washes. 1–2 μg poly A+ RNA was electrophoresed on a 1.5% agarose gel with 2.2 M formaldehyde in PBS and transferred to Nytran N membrane (Schleicher and Schuell, Keene, NH) in 20X SSC, followed by UV cross-linking. A 432 bp probe was amplified by PCR from a mouse *BLyS* cDNA using the following oligos: ZC38343, 5′-GGCAAACAGGCTATTTCTTCAT-3′ and ZC38344, 5′-TTGGAT CAGATTCAACGGGT-3′. The probe was labeled with [α-^{32}P]dCTP using the Rediprime II DNA Labeling System (Amersham Pharmacia Biotech, UK). Hybridization was performed in ExpressHyb (Clontech Laboratories, Palo Alto, CA) for 12 hr at 65°C, followed by four washes in 2X SCC, 0.05% SDS at 22°C and two washes in 0.1X SSC, 0.1% SDS at 50°C.

Cell Staining and Flow Cytometry

Cell suspensions were incubated on ice for 20 min in the presence of the appropriate FITC-, PE-, CyChrome-, PerCP-, and/or biotin-conjugated mAbs in FACS wash buffer (WB, HBSS+1%BSA I-10 mM hepes [pH 7.4]). Cells were washed with 1.5 ml of WB, pelleted, and resuspended in WB containing streptavidin (SA)-CyChrome, if necessary. After another 20 min incubation on ice, the cells were washed and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). RBC and dead cells were excluded by electronically gating data on the basis of FSC versus SSC profiles; a minimum of 2 × 10^4 cells of interest were analyzed further.

Antibodies

Anti-CD3-CyChrome (clone 145-2C11), anti-CD4-PE (clone H129.19), anti-CD5-PE (clone 53-7.3), anti-CD8α-CyChrome (clone 53-6.7), anti-CD19-PE (clone 1D3), anti-CD21/35-FITC (clone 7G6), anti-CD23-PE (clone B3B4), anti-CD43-FITC (clone S7), anti-CD44-PE (clone IM7), anti-CD45R/B220-PerCP (clone RA3-6B2), anti-CD62L-FITC (clone MEL-14), anti-IgD-FITC (clone 11-26c.2a), anti-IgM-biotin (clone R6-60.2), and SA-CyChrome were purchased from PharMingen (San Diego, CA). Goat anti-mouse IgM-PE was obtained from Southern Biotechnology Associates (Birmingham, AL). Rat anti-mouse CD45R/B220-TriColor was purchased from Caltag (Burlingame, CA).

Serum Ig and Anti-Collagen IgG ELISAs

Total serum Igs were measured according to standard ELISA protocols. ELISA microtiter MaxiSorp plates (Nalge/Nunc, Rochester, NY) were coated with 1 μg/ml goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or 1 μg/ml goat anti-mouse IgM (Zymed, South San Francisco, CA) and incubated overnight at 4°C. Plates were blocked 10 min at room temperature with SuperBlock (Pierce, Rockford, IL). Diluted serum samples and Ab standards were added and incubated at RT for 2 hr. Bound Igs were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc or HRP-conjugated goat anti-mouse IgM (Jackson ImmunoResearch). HRP activity was measured with o-phenylenediamine dihydrochloride (OPD) (Pierce), and absorbances were measured on a Spectra MAX 190 at 490 nm (Molecular Devices Corporation, Sunnyvale, CA). For detection of collagen-specific Abs, MaxiSorp plates were coated overnight at 4°C with 5 μg/ml chicken collagen (ELCII kit from Chondrex). Plates were blocked for 10 min at room temperature in SuperBlock. Diluted serum samples were incubated overnight at 4°C. Peroxidase labeled-goat anti-mouse IgG Fc (ICN/Cappel) was used to measure collagen-specific total IgG. HRP activity was measured as described for serum Ig ELISAs. Data are reported as the geometric mean absorbance (OD_{490}).

TACI-Ig Quantitation Assay

To detect soluble TACI-Ig in the serum of mice, MaxiSorp plates were coated overnight at 4°C with a mouse anti-human TACI mAb generated at ZymoGenetics. Plates were washed and blocked for 10 min with SuperBlock prior to the addition of diluted serum samples or TACI-Ig standards. After a 2 hr incubation at 37°C, the plates were washed extensively and incubated for 1 hr at 37°C with HRP-conjugated goat anti-human IgG Fc-specific Abs (Jackson ImmunoResearch). HRP activity was measured with OPD as a substrate. Absorbances were measured at 492 nm.

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